

## Molecular and structural analysis of immunoglobulin E-binding epitopes of Pen ch 13, an alkaline serine protease major allergen from *Penicillium chrysogenum*

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### Summary

**Background** Through proteomic and genomic approaches we have previously identified and characterized an alkaline serine protease that is a major allergen (88% frequency of IgE binding) of *Penicillium chrysogenum* (Pen ch 13).

**Objective** The aim of the present study is to identify the linear IgE-binding epitopes of Pen ch 13.

**Methods** IgE-binding regions were identified by dot-blot immunoassay using 11 phage-displayed peptide fragments spanning the whole molecule of Pen ch 13. The minimal epitope requirements for IgE binding were further defined with overlapping peptides synthesized on derivatized cellulose membranes using SPOTs technology. The critical residues on the immunodominant epitopes were mapped through site-directed mutagenesis. The locations of the IgE epitopes identified were correlated with a three-dimensional structure of Pen ch 13.

**Results** IgE antibodies in 35 serum samples reacted with at least one of the 11 peptide fragments of Pen ch 13. Peptide f-2n (residues 31–61) showed a high-intensity and the highest frequency (77%) of IgE binding. The frequencies of IgE binding to peptide f-4 (residues 93–133), f-1 (residues 1–37) and f-7 (residues 168–206) were 51%, 34% and 31%, respectively. SPOTs assay narrowed down the region of IgE binding of f-2n to residues 48–55 (GHADFGGR). Three, two and one epitope(s) that are four to nine amino acids in length, within f-4, f-1 and f-7, respectively, were found. Site-directed mutagenesis of Pen ch 13 revealed that substitution of His49 and/or Phe52 on Pen ch 13 with methionine resulted in proteins with drastic loss of IgE binding in seven sera tested. Proteins with amino acid replacements at residues 15–18 (RISS), or at residues 112 (I) and 116 (D) have lower IgE-binding reactivity in one of the two patient's sera tested. Substituting residues 117 (W), 119 (V) and 120 (K) also block most of the IgE binding in one of the two patient's sera tested. In addition, replacing residues 203 (V) and 204 (D) along with a deletion at residue 206 (Y) diminished the IgE binding in two serum samples tested. A model was constructed based on the structure of *P. cyclopium* subtilisin protease that has >90% (256 out of 283 amino acids) sequence identity with Pen ch 13. The major epitope (GHADFGGR) on Pen ch 13 formed a loop-like structure and was located at the surface of the allergen.

**Conclusions** Several linear IgE-reactive epitopes and their critical core amino acid residues were identified for the Pen ch 13 allergen. The major linear IgE-binding epitope, 48GHADFGGR55, formed a loop-like structure at the surface of the allergen. Substitution of His49 and/or Phe52 with methionine significantly reduced IgE-binding to Pen ch 13. Mapping of these results on a 3D model of the allergen provides valuable information about the molecular basis of allergenicity for Pen ch 13 and for designing specific immunotherapeutics.

**Keywords** immunoglobulin E epitope mapping, Pen ch 13 allergen, three-dimensional structure

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### Introduction

*Penicillium* and *Aspergillus* species are prevalent indoor airborne fungi that have been identified as causative agents of extrinsic bronchial asthma [1]. At least 18 different

*Aspergillus* allergens have been identified [1]. However, little was known about the *Penicillium* allergens. Our results from studies on fungal allergens showed that the alkaline and/or the vacuolar serine proteases are major allergens of eight prevalent *Penicillium* and *Aspergillus* species (*P. citrinum*, *P. brevicompactum*, *P. chrysogenum*, *P. oxalicum*, *A. fumigatus*, *A. niger*, *A. oryzae* and *A. flavus*) [2–9]. They have been designated as group 13 (alkaline serine protease) and group 18 (vacuolar serine protease) allergens of both fungal genera [10]. Serum IgE antibodies against these fungal allergens were

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detected in more than 80% of atopic asthma patients who were sensitized to *Penicillium* and/or *Aspergillus* species [10].

We have characterized the alkaline serine protease allergens from *P. chrysogenum* (Pen ch 13) [9]. The enzymatic activity of the purified allergen has been assayed. A full-length complementary DNA (cDNA) clone was isolated and heterologous expressed in *Escherichia coli*. Monoclonal antibodies (MoAbs) PCM8, PCM10 and PCM39 reacting with Pen ch 13 have also been generated [6]. Furthermore, IgE cross-reactivity between the group 13 fungal allergens purified from three different *Penicillium* and *Aspergillus* species (Pen ch 13, Pen c 13, Asp fl 13) was demonstrated by an inhibitory ELISA [9].

Environmental allergens can cross-link effector cell-bound IgE antibodies. Subsequently, biologically active mediators that are responsible for the clinical symptoms in allergic disorders are released. We demonstrated that Pen ch 13 can react with human IgE antibodies and induced histamine release from the leucocytes of patients with atopic asthma [9]. Furthermore, Pen ch 13 can induce bronchial hyper-responsiveness and T-helper type 2 cytokine expression in BALB/c mice (manuscript submitted).

Interactions between an allergen and its specific IgE play a critical role in the allergic reaction. Hence, studies of IgE epitopes on allergens will enable us to better understand the molecular interactions between these macromolecules.

The specific aim of this study is to characterize the Pen ch 13 antigenic determinants that are recognized by human IgE antibodies. The sequential IgE epitopes on Pen ch 13 were investigated with phage-displayed overlapping peptides, synthetic peptides attached on membranes and Pen ch 13 proteins carrying substituted amino acid(s). We determined the key amino acid residues within major IgE epitopes and the results were explained with a theoretical Pen ch 13 structural model.

## Materials and methods

### Patient's sera

Sera used in the present study were collected from 35 patients with a clinical history of bronchial asthma and previously shown to contain IgE antibodies against the Pen ch 13 allergen. Serum samples used as controls were collected from non-atopic laboratory volunteers.

### Construction of 11 overlapping Pen ch 13 fragments for phage display and dot-blot immunoassay

The mature Pen ch 13 protein was initially divided into nine overlapping peptide fragments (f-1–f-9; f-1: aa 1–37, f-2: aa 31–82, f-3: aa 52–102, f-4: aa 93–133, f-5: aa 115–152, f-6: aa 137–180, f-7: aa 168–206, f-8: aa 203–250, f-9: aa 240–283) based on the hydrophilicity and the antigenic index of Jameson–Wolf calculated according to the PLOTSTRUCTURE program of the SeqWeb GCG system (version 1.2). Based on preliminary results, two more peptides (f-2n and f-2c; f-2n: aa 31–61, f-2c: aa 52–82) that encompass the region covered by f-2 were also constructed. cDNA fragments corresponding to these Pen ch 13 peptides were amplified with

PCR using 11 sets of gene-specific primers with *Bam*HI and *Eco*RI restriction sites. A full-length cDNA encoding Pen ch 13 is available in our laboratory [9] and used as template in these experiments. The PCR products were restricted with *Bam*HI and *Eco*RI and then inserted into the *Bam*HI and *Eco*RI sites of the T7Select 415–1b vector encoding the capsid protein of bacteriophage T7 (Novagen Inc., Madison, WI, USA). After ligation, the mixture was packaged *in vitro* by mixing 5 µL of the ligation mixture with 25 µL of the T7 packaging extracts. Incubation was carried out at 22 °C for 2 h and the reaction was stopped by adding 270 µL of sterile Luria-Bertani medium (1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5). A plaque assay with *E. coli* BL21 as the host strain was performed to determine the plaque-forming unit (PFU) of the phage solution. To ensure the correct recombinant phages were constructed, DNAs were extracted from individual plaque on agarose plates with 50 µL of 10 mM EDTA (pH 8.0) and heated at 65 °C for 10 min. The lysates (1–2 µL) were then used as templates in PCR experiments. The resulting products were purified and the nucleotide sequences of the cDNA inserts were determined with an ABI 377 automatic sequencer (Applied Biosystem, Foster City, CA, USA).

The correct recombinants were isolated and amplified in liquid culture by infecting *E. coli* BL21 cells ( $OD_{600} = 0.5–1$ ). The infected cultures were incubated by shaking at 37 °C until cell lysis occurred. Cell debris was removed by centrifugation. The titres of the clear lysates were determined by plaque assay and reached at about  $2–5 \times 10^{10}$  PFU/mL. Twelve clear lysates including T7Select415 without insert (as negative control) were dotted individually (5 µL/lysate) onto Duralose-UV Membrane (Stratagene, La Jolla, CA, USA). The membrane was dried at 37 °C for 30 min.

The membranes were blocked with 1% (w/v) skimmed milk, then incubated with diluted human serum samples (1 : 5 dilution in Tris-buffered saline, pH 7.5, containing 0.05% (v/v) Tween 20 and 1% skimmed milk) for 16 h at 4 °C. Bound IgE antibodies were detected with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, CA, USA) as previously described [6–9]. Serum from a normal laboratory volunteer was used as negative control.

### Peptide synthesis and peptide mapping

Fine IgE epitopes within major peptide fragments were defined using the SPOTs kit (Genosys Biotechnologies Inc., Woodlands, TX, USA). Based on the results obtained from dot-blot immunoassay of phage-displayed peptides, overlapping peptides were synthesized on derivatized cellulose sheets with Fmoc chemistry according to the manufacturer's instruction. The synthetic peptides were nine amino acids long and offset by one amino acid. As a control, clear phage lysates previously prepared were dotted on the same membrane along side the synthetic peptides. After drying at 37 °C for 30 min, the membrane was blocked and incubated with pooled sera that previously showed positive IgE reaction against phage-displayed peptide fragments. The membrane was washed, incubated with enzyme-labelled anti-human IgE and substrate solution as described above.

### Site-directed mutagenesis and immunoglobulin E immunoblotting

Amino acids on Pen ch 13 that might responsible for IgE binding were substituted by site-directed mutagenesis (Table 1). The IgE-binding ability of the mutants was compared with that of the wild-type recombinant Pen ch 13. Amino acid substitution was carried out with the Quik Change Site-Directed Mutagenesis Kit (Stratagene). The designated amino acids were replaced with alanine, methionine or glycine (Table 1). The pQE30 double-stranded plasmid DNA (dsDNA) containing the coding sequence of Pen ch 13 was used as template in the PCR and prepared from *dam*<sup>+</sup> *E. coli* strain JM109 using Wizard *Plus* DNA Purification System (Promega, Madison, WI, USA). Fifteen sets of mutagenesis primers were designed and synthesized. Following standard PCR, the parental dsDNA was restricted with *DpnI* and the resulting mixture was transformed into XL1-Blue cells for protein expression. All mutant constructs were confirmed by DNA sequencing.

Wild-type Pen ch 13 and mutants were expressed as 6X His-tagged proteins and purified in denatured form from the Ni-NTA resin columns as described [9]. Purified recombinant proteins were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 µm; Millipore, Bedford, MA, USA). The membranes were blocked with 1% skimmed milk. IgE-binding abilities to wild-type recombinant Pen ch 13 and its mutants were examined as described above.

### Homology-based modelling of Pen ch 13 protein

Molecular modelling of Pen ch 13 was performed with the Modeller program [11] using the structural model of subtilisin

protease from *P. cyclopium* [12] as template. Major IgE epitopes and the critical amino acid residues on the Pen ch 13 model were visualized and displayed with the WebLab Viewer version 3.1 (Accelrys, San Diego, CA, USA).

## Results

### Antibody-binding regions of Pen ch 13

The IgE-binding regions on Pen ch 13 were determined using 35 sera with IgE antibodies against Pen ch 13 and 11 phage-displayed overlapping peptides spanning the whole allergen molecule. We observed that the IgE antibodies in each serum sample tested reacted with at least one of the 11 peptide fragments of Pen ch 13. Representative results using 16 sera are shown in Fig. 1. Among the 35 sera tested, 27 (77%) showed IgE binding to the f-2n fragment (residues 31–61) of Pen ch 13. In addition, IgE binding to this f-2n peptide is relatively intense (Fig. 1). Eighteen sera (51%) reacted with f-4 (residues 93–133), which is the second most common IgE-binding epitope on Pen ch 13. The third and fourth common IgE epitopes are resided in f-1 (residues 1–37), and f-7 (residues 168–206) with frequencies of IgE binding of 34% and 31%, respectively. Fragment f-5 (residues 115–152) has 23% IgE-binding frequency. Fragments f-8 (residues 203–250), f-6 (residues 137–180), and f-3 (residues 52–102) reacted with IgE antibodies in four (11%), three (9%) and one (3%) of these 35 sera, respectively. In this study, 27 sera with IgE reactivity to f-2 (residues 31–82) also showed IgE binding to f-2n, while IgE recognition of f-2c (residues 52–82) and f-9 (residues 240–283) was not detectable among the 35 sera tested. Probably, IgE binding to f-2 in these serum

**Table 1.** Comparison of IgE immunoblot reactivity of wild-type Pen ch 13 and mutants

| Construction of mutants                                                | Patients                | Reduced IgE binding    |                         |
|------------------------------------------------------------------------|-------------------------|------------------------|-------------------------|
|                                                                        |                         | Yes                    | No                      |
| f-1 (1–37)                                                             |                         |                        |                         |
| W11A                                                                   | 2*                      |                        | 2                       |
| K19M                                                                   | 1, 2                    |                        | 1, 2                    |
| <sup>15</sup> RISS <sup>18</sup> to <sup>15</sup> GMAG <sup>18</sup>   | 1, 2                    | 2                      | 1                       |
| f-2n (31–61)                                                           |                         |                        |                         |
| G48A                                                                   | 4, 5                    |                        | 4, 5                    |
| G53A                                                                   | 4, 5                    |                        | 4, 5                    |
| G48A+G53A                                                              | 4, 5                    |                        | 4, 5                    |
| D51A                                                                   | 3, 4, 5, 17, 18, 19, 20 |                        | 3, 4, 5, 17, 18, 19, 20 |
| H49M+F52M                                                              | 1, 3, 4, 5, 17, 18, 21  | 1, 3, 4, 5, 17, 18, 21 |                         |
| H49M                                                                   | 1, 3, 4, 5, 17, 18, 21  | 1, 3, 4, 5, 17, 18, 21 |                         |
| F52M                                                                   | 1, 3, 4, 5, 17, 18, 21  | 1, 3, 4, 5, 17, 18, 21 |                         |
| f-4 (93–133)                                                           |                         |                        |                         |
| <sup>123</sup> KSR <sup>125</sup> to <sup>123</sup> MAA <sup>125</sup> | 7, 20                   |                        | 7, 20                   |
| T128A+K130M                                                            | 7, 20                   |                        | 7, 20                   |
| W117G+V119A+K120A                                                      | 7, 20                   | 7                      | 20                      |
| I112M+D116A                                                            | 7, 20                   | 7                      | 20                      |
| f-7 (168–206)                                                          |                         |                        |                         |
| V203A+D204A+Y206 deletion                                              | 9, 10                   | 9, 10                  |                         |
| V203A                                                                  | 9, 10                   |                        | 9, 10                   |

\*Sera from patients 1–5, 7, 9, 10 are the same as those shown in Fig. 1, respectively. IgE antibodies in serum nos. 17–19 and 21 reacted mainly with f-2n and f-2 (31–82). IgE antibodies in serum no. 20 reacted with f-4 and weakly with f-2n and f-2.

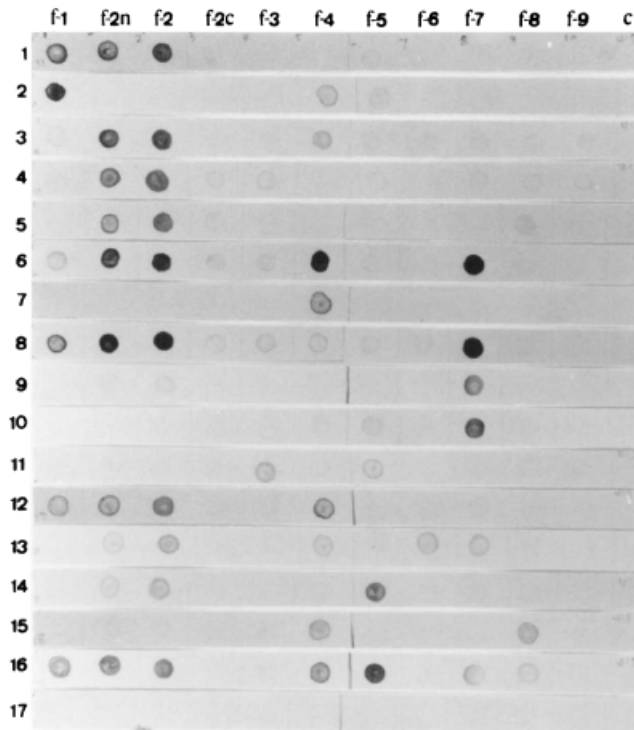


**Fig. 1.** Representative results of dot immunoassay showing reactivity of IgE antibodies to 11 phage-displayed peptide fragments of Pen ch 13. Strips 1–16 show the results obtained using sera from 16 asthma patients (patient nos. 1–16) with IgE antibodies against Pen ch 13. Strip 17 was a control experiment using serum from a healthy volunteer. Dot c on each strip represented the phage coat protein and acted as control.

samples originated from their reactivity to f-2n. IgE antibodies in the serum from a healthy volunteer do not react with the Pen ch 13 peptide fragments (Fig. 1, strip 17). In addition, all the serum samples tested showed negative reactivity to the coat protein of the T7 bacteriophage as shown in Fig. 1, spot c of all strips.

#### *Immunoglobulin E epitopes on Pen ch 13 mapped by SPOTs assay*

The four most common IgE-reacting peptide fragments of Pen ch 13, according to the IgE-binding frequency, are f-2n (residues 31–61), f-4 (residues 93–133), f-1 (residues 1–37) and f-7 (residues 168–206). The IgE-binding regions on these fragments were further narrowed down with the SPOTs assay.

For each fragment, 22 or 23 overlapping peptides were synthesized on cellulose membranes. The overlapping peptides are nine amino acids long and offset by one residue. Overlapping peptides covering amino acid residues 1–31 (f-1'), 32–61 (f-2n'), 103–133 (f-4'), and 172–203 (f-7'), of peptide fragments f-1, f-2n, f-4 and f-7, respectively, were constructed (Fig. 2). Pooled sera from individuals with positive IgE binding to the corresponding Pen ch 13 peptides analysed in the phage display study were used to map the fine linear IgE epitopes. Serum nos. 1 and 2, serum nos. 3–5, serum nos. 6 and 7, and serum nos. 8–10, as shown in Fig. 1, were reacted with peptide fragments f-1', f-2n', f-4' and f-7', respectively. Representative results of the SPOTs immunoassay for f-1' and f-4' are shown in Fig. 3. A summary of the

#### Fragment-1': aa 1–31

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ANVVQRNAPPSWGLSRISSKKSGATDYVYDST
p7 (7–15)      NAPSWGLSR
p8 (8–16)      APSWGLSRI
p9 (9–17)      PSWGLSRI
p10 (10–18)    SWGLSRISS
p11 (11–19)    WGLSRISSK
p12 (12–20)    GLSRISSKK
p13 (13–21)    LSRISSKKS
p14 (14–22)    SRISSKKSG
p15 (15–23)    RISSKKSG
p16 (16–24)    ISSKKSGAT
p17 (17–25)    SSKKSGATD

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#### Fragment-2n': aa 32–61

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AGEGIVIIYGVDTGIDIGHADFGGRAEWGNTN
p15 (46–54)      DIGHADFGG
p16 (47–55)      IGHADFGGR
p17 (48–56)      GHADFGGRA
p18 (49–57)      HADFGGRAE

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#### Fragment-4': aa 103–133

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DGSGTNSQVIAGMDWAVKDSKSRGATGKSVM
p8 (110–118)    QVIAGMDWA
p9 (111–119)    VIAGMDWAV
p10 (112–120)   IAGMDWAVK
p11 (113–121)   AGMDWAVKD
p12 (114–122)   GMDWAVKDS
p13 (115–123)   MDWAVKDSK
p14 (116–124)   DWAVKDSKS
p15 (117–125)   WAVKDSKSR
p16 (118–126)   AVKDSKSRG
p17 (119–127)   VKDSKSRGA
p18 (120–128)   KDSKSRGAT
p19 (121–129)   DSKSRGATG
p20 (122–130)   SKSRGATGK
p21 (123–131)   KSRGATGKS
p22 (124–132)   SRGATGKSV
p23 (125–133)   RGATGKSVM

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#### Fragment-7': aa 172–203

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NSSPASAPNVCTIAASTNSDGSSFTNFGSVV
P23 (194–202)    ASSFTNFGSV
P24 (195–203)    SSFTNFGSVV

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**Fig. 2.** Summary of IgE-binding epitopes of four phage-displayed peptide fragments from Pen ch 13 mapped by SPOTs assay. For each peptide fragment, 22 or 23 overlapping peptides were synthesized. The synthetic peptides are nine amino acids long with overlapping sequences offset by one residue. The SPOTs membranes were incubated with pooled serum from patients with positive IgE binding to the corresponding Pen ch 13 fragments analyzed in the phage-display study. IgE-reacting synthetic peptides are listed and shown in bold type. Common sequences of adjacent peptides recognized by IgE antibodies are shaded. The common IgE-binding peptide sequences within the Pen ch 13 fragments are boldfaced, underlined and shaded.

SPOTs assay of all four IgE-reacting peptides is shown in Fig. 2. Our results indicate that IgE reacts with <sup>48</sup>GHADFGGR<sup>55</sup> and <sup>195</sup>SFTNFGSVV<sup>203</sup> of f-2n' (aa 32–61) and f-7' (aa 172–203), respectively (Fig. 2). Two epitopes (<sup>9</sup>PSWGLSR<sup>16</sup> and <sup>16</sup>ISSK<sup>19</sup>) were identified in f-1' (aa 1–31) while f-4' (aa 103–133) has three IgE-interacting regions (<sup>112</sup>IAGMDWAV<sup>119</sup>, <sup>120</sup>KDSKSR<sup>125</sup> and <sup>125</sup>RGATGK<sup>130</sup>).

#### *Immunoglobulin E reactivity to recombinant Pen ch 13 mutants*

To gain further insights into the IgE-binding epitopes of Pen ch 13, residues identified with the SPOTs assay were substituted with site-directed mutagenesis. The mutated proteins were then interacted with sera from patients with positive IgE binding to the corresponding Pen ch 13 peptides analysed in the phage display study (Table 1). The IgE reactivity against recombinant Pen ch 13 and its mutants was

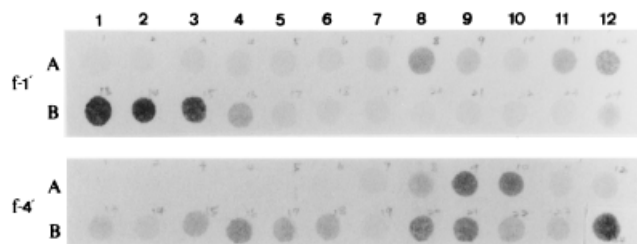


**Fig. 3.** Representative results of SPOTs analysis of fine IgE epitopes within Pen ch 13 fragments f-1' (aa 1–31) and f-4' (103–133). Linear peptides of nine amino acids were synthesized on derivatized cellulose membrane with sequences offset by one amino acid. Dots A1–A12 and B1–B12 contained synthesizing peptides p1–p12 and p13–p23, respectively. Phage-displayed peptide fragment f-1 (aa 1–37) and f-4 (93–133) of Pen ch 13 were dotted on spot position B12 of individual membrane as positive control. Serum pools of serum nos. 1 and 2 and serum nos. 6 and 7 in Fig. 1 were used in probing IgE reactivity with peptides synthesized according to sequences of f-1' and f-4', respectively.

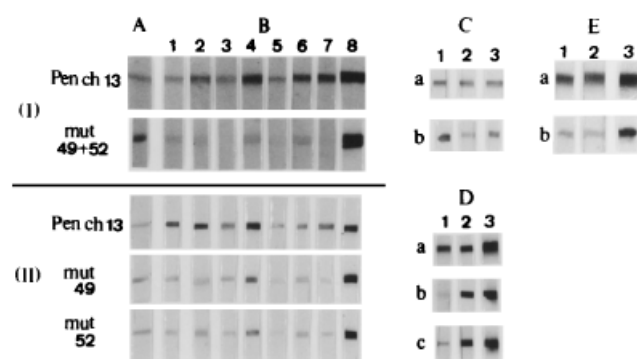


**Fig. 4.** IgE immunoblotting reactivity to wild-type recombinant Pen ch 13 and site-directed mutants. Parts I and II represent double mutation directed at His49 plus Phe52 located within peptide fragment f-2n (aa 31–61) and single mutation at either amino acid residue, respectively. Strips in panel A show Coomassie blue-stained protein profiles of recombinant Pen ch 13 proteins. Strip nos. 1–7 in panel B represent results of immunoblotting using sera from patients 1, 3, 4, 5, 17, 18, 21 (Table 1), respectively. Strip no. 8 represents a control by using serum from patient no. 2 (Table 1) with IgE reactivity to peptide fragment f-1 (aa 1–37) of Pen ch 13 (Fig. 1). Strips in panels C–E show IgE immunoblots to the wild-type Pen ch 13 (part a) and mutant Pen ch 13 proteins directed at residues within peptide fragments f-1 (aa 1–37) (15–18, part b), f-4 (aa 93–133) (117, 119 plus 120– part b, and 112 plus 116– part c) and f-7 (168–206) (203, 204 and 206, part b) (Table 1), respectively. Strips 1 and 2 in panels C–E represent results using sera from patients 1 and 2, 7 and 20, as well as 9 and 10, respectively, as shown in Table 1. Strip 3 in panels C–E represent controls by using serum nos. 19 (panel D) and 20 (panels C and E).

then compared. Results obtained are summarized in Table 1. Immunoblotting data of Pen ch 13 mutants with changes in IgE binding are shown in Fig. 4. Among the eight residues (48–55) identified within f-2n (aa 31–61), we have mutated five positions singly or in combination. Pen ch 13 with His49 or Phe52 to methionine replacement (His49Met and Pen52Met mutants, respectively) showed a decrease in IgE-binding intensity in seven sera with IgE antibodies against f-2n (Fig. 4, panel B of part II). A mutant with substitution at both residues caused a dramatic loss of IgE reactivity (Fig. 4, panel B of part I). However, replacements at Gly48, Asp51 or Gly53 have no effect on IgE binding. For control, decrease in IgE binding to His49Met and/or Phe52Met mutants was not detectable when serum no. 2 with IgE antibodies against peptide fragments f-1 (aa 1–37) and f-4 (aa 93–133) (Fig. 1) was used (Fig. 4, strips 8 of panel B in parts I and II).

In SPOTs analysis, two IgE epitopes covering residues 9–16 and 16–19 on f-1' (aa 1–31) were identified. Replacement at residue 11 or 19 had no effect on IgE binding when serum no. 2 or serum nos. 1 and 2, respectively, were tested (Table 1). Pen ch 13 mutant constructed by replacing <sup>15</sup>RISS<sup>18</sup> with GMAG resulted in a decrease in the IgE-binding reactivity to Pen ch 13 of patient no. 2 (Fig. 4, panel C, strip b-2).

We have identified three epitopes covering 19 residues on f-4' (aa 103–133) by SPOTs immunoassay. The first epitope counting from the N-terminal has eight residues (112–119). We made replacements at four positions within this region plus substituting Lys120 from the second epitope with an alanine (<sup>112</sup>IAGMDWAVK<sup>120</sup>) and accomplished only a reduction in the IgE-binding reactivity of patient 7 (Fig. 4, panel D, strips b-1 and c-1). We have substituted a stretch of three amino acids (residues 123–125) and generated a double mutation (Thr128 to Ala and Lys130 to Met) within the second (<sup>120</sup>KDSKSR<sup>125</sup>) and third (<sup>125</sup>RGATGK<sup>130</sup>) epitopes, respectively. These mutants did not change the IgE-binding reactivity of the patients.

The SPOTs analysis revealed that IgE reacted only to <sup>195</sup>SFTNFGSVV<sup>203</sup>, the extreme C-terminal of f-7' (aa 172–203) (Fig. 2). Hence, we cannot exclude the possibility that the IgE-binding region extends beyond Val203 of f-7'. Therefore, we constructed Pen ch 13 mutants with Val203 to Ala or both Val203 and Asp204 to Ala plus a Tyr206 deletion. For patients 9 and 10 with IgE reactivity to f-7 (aa 168–206), mutation at Val203 and Asp204 plus a deletion of Tyr206 induced a decrease in the IgE reactivity (Fig. 4, panel E, strips b-1 and b-2, respectively). For control, serum no. 20 with IgE antibodies against f-2n (aa 31–61) and f-4 (aa 93–133) were used (Fig. 4, panel E, strips a-3 and b-3).

### Homology modelling

In this study, a homology-based model of the tertiary structure of Pen ch 13 was generated to display the location of the epitopes on this major fungal allergen. The four most frequent functional IgE-binding epitopes are located on the surface of the protein molecule (Fig. 5, panel a). Two of them (f-2n: 48–55 and f-7: 195–206) form a loop-like structure and the other two (f-1: 9–19 and f-4: 112–130) are combinations of helices and bends that connect secondary structures (Fig. 5, panel b).

### Discussion

In the present study, the molecular and structural natures of allergenicity of the major Pen ch 13 allergen from *P. chrysogenum* were analysed.

Our results showed that although human IgE antibodies reacted with antigenic determinants distributed throughout the whole molecule, a major IgE-binding region was located at a region (residues 48–55) close to the N-terminus of Pen ch 13. In addition, heterogeneity in IgE responses to Pen ch 13 in terms of antibody specificity and intensity in individual patient was also observed. It correlates with the finding that most allergens have multiple IgE-binding sites and it is at least partly the result of the polyclonal nature of the immune response to protein allergens [13–15].

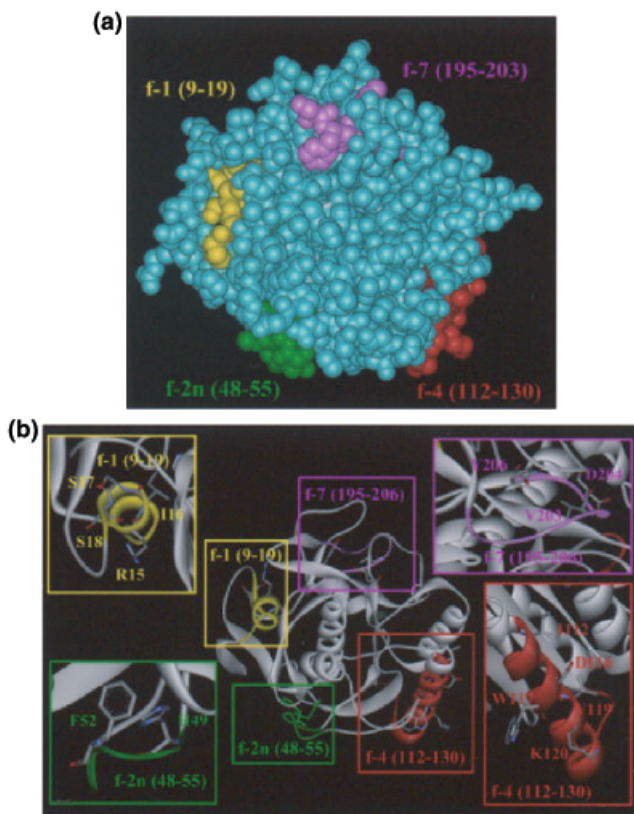


**Fig. 5.** (a) A space-filled model representing the three-dimensional structure of Pen ch 13. The four most frequent functional IgE epitopes (f-2n, f-4, f-1 and f-7) defined by phage display and SPOTs array are located on the protein surface and coloured in green, red, yellow and magenta, respectively. (b) Ribbon diagram of Pen ch 13 showing these sequential epitopes and their respective critical amino acid residues involved in IgE binding. The model was constructed with Modeller using the coordinates in the pdb file 4TST as template, viewed and displayed with WebLab Viewer (Accelrys).

To further localize functional epitope(s) within IgE-reacting fragments, SPOTs assay using synthetic overlapping peptides was performed. Separate epitopes within the same IgE-binding region can be identified by observing the changes in intensity of the sequence overlapping peptides against the same batch of antibodies. We found that one determinant each was located in f-2n' (aa 32–61) or f-7' (aa 172–203). However, two and three overlapping antibody-binding sites were delineated in f-1' (aa 1–31) and f-4' (aa 103–133), respectively. Multiple linear IgE-binding epitopes residing within an allergenic peptide have been observed in studies of other allergens, such as in Hol 1 5 [16]. Our results also show that these functional epitopes are four to nine amino acids in length, affirming the finding of previous report that linear epitopes are typically five to 10 residues in length [17–19]. Results obtained from identification of IgE epitopes may enable the design of monovalent peptide-based therapeutic approaches that may be used in blocking the allergen-induced histamine release or active immunization to induce the blocking IgG antibodies.

The structures of serine proteases of the subtilisin family have been known for decades [20, 21]. Among these serine proteases with known structures, Pen ch 13 has the highest sequence identity with the major extracellular protease from

the fungus *P. cyclopium* [12]. The amino acid sequences of Pen ch 13 and the *P. cyclopium* protease share >90% sequence identity (256 out of 283 amino acids). Noticeably, among the N-terminal 230 amino acids, Pen ch 13 differs from the *P. cyclopium* protease in only four places (residues 6–8 and Trp58 to Thr substitution). Furthermore, among these four changes, one of them (Arg6 to Lys) is a conservative substitution. In addition, the sequence of *P. cyclopium* protease is only 49% identical to that of proteinase K of *Tritirachium album* [22] and the three-dimensional structures of these two proteins are virtually identical [12]. Therefore, the structure of Pen ch 13 (Fig. 5) can be inferred from the *P. cyclopium* protease model. The folding topology and to some extent, the orientation of the side-chains at the N-terminal of these two proteases should be similar.

Among the four IgE-binding regions on Pen ch 13, the epitopes on f-2n (<sup>48</sup>GHADFGGR<sup>55</sup>) and f-4 (<sup>112</sup>IAGMD-WAV<sup>119</sup>, <sup>120</sup>KDSKSR<sup>125</sup> and <sup>125</sup>RGATGK<sup>130</sup>) have the highest binding frequency. These two regions are on the surface of the protein molecule (Fig. 5, panel a) and accessible for interaction with Ig. Residues 48–55 of f-2n are part of a coil that connects two  $\beta$  sheets,  $\beta$ III and  $\beta$ II2 (helixes and  $\beta$  sheets are designated according to Betzel et al. [22]) (Fig. 5, panel b). Within this octapeptide, we have generated site-directed mutants at Gly48, His49, Asp51, Phe52 and Gly53 (Table 1). The purified wild-type recombinant protein and mutants were transferred onto PVDF membranes after SDS-PAGE and the IgE-binding ability was assessed. The structural similarity between the proteins in solution and those on the blot is difficult, if not impossible to determine. However, the glycine residues (Gly48 and Gly53) do not have side-chains that interact with others while the only hydrogen bond observed for Asp51 is that with the main-chain amide of Phe86. Mutations performed on these residues probably cause minimal perturbation of the structure and do not decrease the IgE binding activity of the protein analyzed using sera from two patients as shown on Table 1. The imidazole side chain on His49 only forms hydrogen bonds with the main-chain oxygen of Asp51 and Gly220. Major structural changes on Pen ch 13 are not expected with substitution of this amino acid. However, decrease in IgE-binding activity was observed for the His49 to methionine mutant. The result implies that His49 plays a role in IgE recognition at least in sera from the seven patients analysed.

The Phe52 side chain is not exposed but pointed towards the inside of the protein molecular. The phenyl ring at residue 52 cannot form hydrogen bondings but we cannot exclude the possibility that it forms van der Waals interactions with nearby aliphatic side chains (Ile45, Ile47, Ala56, Ala78 and Val94). We observed a decrease in IgE binding of the Phe52 mutant (Table 1) after SDS-PAGE separation and electroblotting, and concluded that this residue is needed for IgE recognition of these patients. It is also of interest to find that His49 and Phe52 residues are highly conserved in serine protease allergens (Pen ch 13, AF084546; Pen ch 13, AF321100; Asp f 13, P28296; Asp o 13, P12547; Pen ch 18, AF098517; Pen ch 18, AF263454; Pen o 18, AF243425; Asp f 18, Y13338; Asp n 18, P33295) and could contribute to the allergenic cross-reactivity among these important fungal allergens.

There are three epitopes on peptide f-4 (aa 93–133). The first two (<sup>112</sup>IAGMDWAV<sup>119</sup> and <sup>120</sup>KDSKSR<sup>125</sup>) are located on the  $\alpha$ 3 helix (Fig. 5, panel b). This region is again on the surface of the protein (Fig. 5, panel a). Mutations on the  $\alpha$ 3 helix (Ile112 to Met and Asp116 to Ala double mutant; Trp117 to Gly, Val119 to Ala and Lys120 to Ala triple mutant) reduced only the IgE-binding in the serum of patient no. 7. The  $\alpha$ 3 helix is connected to the  $\beta$ II4  $\beta$  sheet with a five residues (<sup>126</sup>GATGK<sup>130</sup>) bend. Two Gly residues are located on the bend to facilitate the turning of the carbon backbone. The Thr128 to Ala and Lys130 to Met double mutant that we generated retain the same IgE-binding activity as that of the wild-type recombinant protein analyzed using sera from two patients as shown on Table 1.

There are two and one epitope(s) on peptide f-1 (aa 1–37) and f-7 (168–206), respectively. These regions are on the surface of Pen ch 13 (Fig. 5, panel a) and form a helix (f-1) or a loop-like configuration (f-7) (Fig. 5, panel b). In a homology-based structural model of glycinin G2, five of the six IgE-binding regions in this soybean allergen are located on the surface of the trimer model [23]. In addition, IgE epitope regions located in the loop and helix areas as identified in this study were also found in Par j 1 and Par j 2 pollen allergens [24].

Results from mutational analysis of IgE-binding epitopes have revealed that single amino acid changes within these peptides could lead to a reduction or loss of IgE binding [13, 19, 25]. In addition, allergen mutants directed at amino acid residues residing within IgE epitopes were also found to result in a reduced IgE-binding activity [26, 27]. Thus, results obtained from the present study and those as examples mentioned above will enable an improved therapeutic approach in the design of hypoallergenic variant allergen molecules.

In conclusion, despite the rapidly increasing number of recombinant allergens, relatively few IgE-reactive B cell epitopes have been defined and characterized at the molecular and structural bases [23, 24, 28, 29]. In this study, although the method we used maps only linear epitopes, the strategies described in this study allowed us to identify residues 48–55 on Pen ch 13 as an immunodominant epitope. By correlating our immunological data with the structural model of the protein, this immunodominant epitope is a long flexible loop located on the protein surface. Other minor epitopes (<sup>125</sup>RGATKG<sup>130</sup>, <sup>16</sup>ISSK<sup>19</sup> and <sup>195</sup>SFTNFGSVV<sup>203</sup>) at least are part of a bend or loop that connects stable secondary structures. Although, until now, no common structural characteristics of linear IgE epitopes and allergen molecules have been identified [15, 30], our results obtained from the present study provide more information on the molecular and structural features of allergenicity of protein allergens and bases for developing effective therapeutic strategies.

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